TRANSGENIC CIRCULATING ENDOTHELIAL CELLS

BACKGROUND OF THE INVENTION

This invention was made with the support of NIH Grant No. HL 55174 (now HL 30160). The Government has certain rights in the invention.

The endothelial cell participates in numerous functions of vascular physiology. Many factors, such as cytokines, can alter the surface of the endothelial cell and thereby modulate the role of the endothelium in coagulation, inflammation, vaso-regulation, and adhesion. See, for example, R. P. Hebbel et al., J. Lab. Clin. Med., 129, 288 (1997); J. S. Pober, Am. J. Path., 133, 426 (1988); E. J. Favaloro, Immunol. Cell. Biol., 71, 571 (1993). The endothelial cell may also have a key role in the vascular pathology of sickle cell anemia, including the vaso-occlusions that cause acute painful crises. However, research in this area has been hindered by the inaccessibility of vascular endothelium in patients. For example, E. M. Levine et al. (U.S. Pat. No. 5,132,223) disclosed cloning and serial cultivation of adult human endothelial cells derived from brain-dead, but heart-beating cadaver organs. K. Gupta et al., Exp. Cell. Res., 230, 244 (1997) reported the culture of microvascular endothelial cells derived from newborn human foreskin. Thus, circulating endothelial cells might provide useful material for the study of vascular pathologies, for gene therapy, and for biomedical engineering applications. In previous investigations increased numbers of circulating endothelial cells have been found in sickle cell anemia and other conditions associated with vascular injury, such as that due to cytomegalovirus infection, rickettsial infection, myocardial infarction, intravascular instrumentation, and endotoxinemia. See, for example, F. George et al., Blood, 80, Suppl: 12a, abstract (1992); E. Percivalle et al., J. Clin. Invest., 92, 663 (1993), F. George et al., Blood, 82, 2109 (1993); C. A. Bouvier et al., Thomb. Diath. Haemorrh. Suppl., 40, 163 (1970); F. George et al., J. Immunol. Meth., 139, 65 (1991) and R. G. Gerrity et al., Exp. Mol. Pathol., 24, 59 (1976).

However, in normal donors, there are only about 2-3 circulating endothelial cells per ml of blood; they have a quiescent phenotype, and about 50% of them are microvascular as evidenced by CD36 positivity. See, A. Solovey et al., New Engl. J. Med., 337, 1584 (1997), who reported using the methodology of Gupta et al., cited above, to coculture viable circulating endothelial cells identified in the blood of patients with sickle cell anemia with primary microvascular endothelial cells (MVEC). T. Asahara et al., Science, 275, 964 (1997) isolated putative endothelial cell (EC) progenitors from human peripheral blood after $CD34^+$ enrichment by magnetic bead selection on the basis of cell surface antigen expression. The cells were cultured on fibronectin-coated wells in modified M-199 medium containing bovine brain extract and 20% fetal bovine serum. Q. Shi et al., Blood, 92, 362 (1998) characterized bone marrow-derived precursor endothelial cells by isolating $CD34^+$ cells derived from peripheral blood using murine anti-$CD34^+$ antibody binding followed by exposure to anti-mouse immunomagnetic beads. The cells were cultured in gelatin or fibronectin-coated plastic wells in M199 medium containing VEGF, FBS, bFGF and IGF.

However, due to the low concentration of CEC in blood, a need exists for a culture method and medium that will permit the rapid expansion of CEC from blood, without the attendant difficulties of isolation discussed above.

SUMMARY OF THE INVENTION

The present invention provides a process for expanding the population of endothelial cells (EC) present in an aliquot of peripheral mammalian blood, i.e., blood obtained from an animal or human patient. The process comprises culturing buffy coat mononuclear cells, which are readily obtained from peripheral mammalian blood. The buffy coat mononuclear cells are cultured in contact with a surface coated with collagen I, such as a coated plastic culture well, wall of a culture flask or bioreactor, in a culture medium containing an effective amount of vascular endothelial growth factor (VEGF), which medium is free of bovine brain extract. Optionally, the buffy coat mononuclear cells can thereafter be cultured in contact

with a surface coated with fibronectin/gelatin. The medium can also comprise heparin and/or dextran sulfate, and other growth factors conventionally employed in endothelial cell culture media. The present method accomplishes the rapid and extensive expansion of the initial population of endothelial cells and any endothelial progenitor cells present in the population of buffy coat mononuclear cells. For example, the present method typically results in an outgrowth of endothelial cells that is at least a billion-fold greater than the 20-30 endothelial cells identifiable on culture day #2, when endothelial cells are first counted. The endothelial outgrowth includes the expansion of two populations. There is a limited expansion of the mature endothelial cells of vessel wall origin that are found in fresh blood, as well as a delayed, but greater, expansion of a rarer population of marrow-derived cells, e.g., endothelial progenitor cells or angioblasts, that are also found in fresh blood.

Unexpectedly, the cultured endothelial cells have been found to be amenable to cryopreservation in conventional cryopreservation media, followed by thawing and continued culture/expansion. The present method is thus more convenient and less complex than methods based on processing and culturing adult vessel human endothelial cells, as described, for example, in E. M. Levine et al. (U.S. Pat. No. 5,132,223). It does not comprise selection of sub-populations of EC-enriched hematopoietic cells using antibodies and/or magnetic bead technology.

The present invention also comprises an isolated, purified transgenic mammalian endothelial cell comprising a recombinant DNA sequence encoding at least one biologically active preselected protein or polypeptide, such as a Factor VIII protein, and optionally, a selectable marker gene or reporter gene. Preferably, the transgenic endothelial cell is prepared by stably transforming (transfecting/transducing) a population of circulating endothelial cells outgrown from blood in accord with the present method, with a vector comprising an isolated DNA sequence encoding the protein/polypeptide of interest, operably linked to a promoter functional in said endothelial cells.

A population of said transgenic cells can be formulated into a pharmaceutical composition and administered to a mammal, such as a human patient

afflicted with hemophilia, preferably in combination with a pharmaceutically acceptable carrier. The carrier may be a liquid carrier (for example, a saline solution) or a solid carrier; e.g., an implant. In employing a liquid carrier, the engineered cells may be introduced, e.g., intravenously, sub-cutaneously, intramuscularly, intraperitoneally, intralesionally, and the like. The polypeptide, such as Factor VIII protein or proteins are expressed *in situ*, i.e., in the bloodstream of said mammal in an amount effective to treat (alleviate) said hemophilia. Since the transgenic endothelial cells will also migrate to the bone marrow, they are also useful for many aspects of gene therapy, apart from treating circulatory pathologies. Other uses of these transgenic EC, such as in biocompatibilization of implantable devices, diagnostics, and local drug delivery, are discussed hereinbelow.

Novel vectors useful for transforming the expanded circulating endothelial cells of the invention are also within the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph plotting the outgrowth of endothelial cells in accord with the method of the invention. Arrows show times at which cultures were passaged. All data points are plotted as mean ± SD. Data are shown for n = 5 through culture passage 6, and n = 4 for subsequent passages.

Figure 2 is a graph plotting the outgrowth of endothelial cells that were subjected to cryopreservation.

Figure 3 depicts the DNA sequence of HSQ/eGFP (SEQ ID NO:1). (HFVIII/SQ/egfp) which comprises original HB with B-domain SQ insert based on Lind et al., Eur. J. Biochem., 232; and p21 primer sequence containing eGFP protein sequence.

Figure 4 shows the DNA sequence (SEQ ID NO:2) of HSQRENeo.

Figure 5 shows the results of G418 selection (shaded area) of outgrowth endothelial cells stably transfected with a vector encoding eGFP.

Figure 6 shows factor VIII activity in conditioned media from cells transfected with various constructs (see Example 7).

Figure 7 depicts the results of a RT-PCR analysis for human FVIII mRNA in cells transfected with various constructs.

DETAILED DESCRIPTION OF THE INVENTION

The endothelial cell culture medium used in the process of this invention comprises any of the media conventionally employed for the culture of this type of cell, as modified in accord with the present method, to include an effective amount of endothelial cell growth factor (ECG or VEGF), and to exclude bovine brain extract. The exclusion of bovine brain extract is advantageous both from a standpoint of consistency of formulation, and from a health standpoint, in that cells are not subjected to the risk of contamination with infectious agents (viruses, prions, etc.) which may be present in such extracts.

Preferably, the medium contains heparin, dextran sulfate or a combination thereof. These materials are described in detail in U.S. Pat. No. 5,132,223. Of the various basal endothelial cell growth media, EGM®-2 (Clonetics, Inc., San Diego, CA) is particularly preferred. It is based on CCMD®130 culture medium plus human epidermal growth factor (hEGF), human basic fibroblast growth factor (hFGF-B), human recombinant insulin-like growth factor (Long R3-IGF-1). FGF can be present at about 0.5-5 ng/ml, EGF can be present at about 0.5-10 ng/ml and IGF can be present at about 1-7.5 ng/ml of the culture medium.

The medium can also comprise hydrocortisone (0.1-2 µg/ml), heparin (1-20 µg/ml), gentamicin, amphotericin-B (0.1-0.5 mg/ml) and fetal bovine serum. Vascular endothelial growth factor (VEGF) is present at about 1-100 ng/ml, preferably about 5-25 ng/ml. It is included within this concentration range in some commercially available media or supplements or is available from Collaborative Research, Inc. VEGF can also be prepared as described by Maciag et al., PNAS USA, 76, 5674 (1979).

A medium that is effective to disperse and pre-wash the buffy coat mononuclear cells prior to culturing, has been described by K. Gupta et al., Exp. Cell Res., 230, 244 (1997). This endothelial culture medium consists of MCDB 131 medium supplemented with 1 μg/ml hydrocortisone acetate, 5×10^{-4} M bibutyryl cAMP, 1.6 nM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 0.25 mg/ml amphotericin B, 0.004% heparin and 20% human male serum. VEGF (0.01-100 ng/ml) can also be added to this medium.

Circulating endothelial cells can be isolated from whole blood via isolation of the buffy coat mononuclear cells by methodologies well-known to the art. For example, see A. Solovey et al., New Engl. J. Med., 337, 1584 (1997). Whole blood is anti-coagulated and diluted with a physiological salt solution containing EDTA and BSA. The diluted blood is layered onto Histopaque® 1077 (Sigma Chem. Co.) and centrifuged at ambient temperature. The buffy coat mononuclear cells are collected and washed by centrifuging with the medium described in K. Gupta et al., Exp. Cell. Research, 230, 244 (1977), described hereinabove, i.e., supplemented MCDB 131 medium.

The buffy coat mononuclear cells are resuspended in the endothelial cell culture medium (i.e., EGM®-2) and held in a suitable vessel having the walls thereof coated with collagen I. Type I collagen is commercially available from Sigma Chem. Co. (St. Louis, MO) and is obtainable from bovine Achilles tendon, calf skin, rat tail, and human placenta. See, for example, Niyiloizi et al., J. Biol. Chem., 259, 14170 (1984). On day two of culture, unattached buffy coat mononuclear cells and cell debris are removed when the medium is changed, and the small number of adhered cells exhibit endothelial cell morphology, and stain positive with mAb P1H12.

Cultured cells were evaluated by inverted phase-contrast microscopy to confirm the characteristic cobblestone morphology of the endothelial cells and to examine for the presence of cells other than endothelial cells. Cells were also analyzed for von Willebrand factor (vWF) and CD36 expression by immunofluorescence and flow cytometry. The presence of cell surface CD36 has

been used as a marker for human microvascular endothelial cells (HDMEC), as it has been shown that most MEC express CD36, but endothelial cells lining large vessels do not. Briefly, cells in suspension were fixed with paraformaldehyde, cytospun onto glass slides, washed, and incubated with either rabbit anti-human vWF (1:400 dilution) or anti-CD36 mAb FA6-152 (5 μg/ml) (Immunotech, Westbrook, ME). Slides were then washed and incubated with secondary antibodies conjugated to rhodamine or fluorescein, washed, and viewed under a fluorescent microscope.

To identify endothelial cells, the antibody P1H12 was also used. This murine IgG1 monoclonal antibody was obtained by immunizing mice with HUVEC, generating a hybridoma line, and separating IgG from supernatants of hybridoma cell cultures with a protein G column. For some studies, fluorescein isothiocyanate-labeled P1H12 was used, prepared with the Fluoro Tag FITC Conjugation kit (Sigma).

P1H12 reacts specifically with endothelial cells in blood. It stains primary HUVEC and MVEC cultures and the endothelial cells of all vessels in frozen sections of human skin, intestine, ovary, tonsil, lymph node, lung, and kidney. It does not stain any other type of cell in those tissues. It does not stain carcinoma cell lines HT-29 and COLO205, melanoma cells lines A-375 and M21, the T-cell lines Jurkat and HuT78, fibroblasts, HL-60 or Chinese-hamster-ovary cells, or Epstein-Barr virus-transformed B-cell lines. It does not stain monocytes, granulocytes, red cells, platelets, T cells, or B cells from marrow or peripheral blood; nor does it react with marrow megakaryocytes or the megakaryoblast line HU3. The peripheral blood cells that do stain with P1H12 are also positive for both von Willebrand factor and thrombomodulin (the combined expression of which is limited to endothelium), and they stain for flt and flk (receptors for the endothelial-specific vascular endothelial growth factor). Subgroups of P1H12-positive blood cells also stain for CD34 and two endothelial-specific activation markers (VCAM and E-selectin).

After good EC growth is obtained, preferably after about 10^3-fold expansion, or at 15-25 days, the cells are trypsinized and isolated from the supernatant by

centrifugation. The cells are then suspended in the initial culture medium in a flask coated with fibronectin/gelatin for continued culture. Clones can be derived from secondary cultures and seeded at about 10 cells/cm^2 of flask surface. The clones are then serially propagated in the culture medium.

It was also found that EC cultured in accord with the present method can be cryopreserved and then thawed and returned to culture without significant loss of their capacity to proliferate. To cryopreserve the cells, the cultured cells can be detached as described hereinabove and resuspended in suitable cryopreservation medium, i.e., containing a cryopreservation agent such as sugar(s), BSA, dimethylsulfoxide (DMSO), glycerol, glycerol esters and the like.

Cryopreservation of hematopoietic cells, such as bone marrow and peripheral blood fractions enriched in progenitor stem cells, has become standard clinical practice for autologous bone marrow transplantation (Areman et al., Bone Marrow and Stem Cell Processing: A Manual of Current Techniques, F. A. Davis, Philadelphia, PA, 1st edition (1992)). Two basic techniques are used to cryopreserve hematopoietic cells. The most commonly used technique in clinical laboratories, uses tissue culture medium combined with 95% fetal calf serum ("FCS"), and 5 v/v % dimethylsulfoxide ("DMSO"). After suspension in the cryopreservation medium, the cells are placed on ice for about 5 minutes, then at -70° C overnight and finally in liquid nitrogen. This technique was developed in the early 1960s by Ashwood-Smith (see Sputtek et al., Clinical Applications of Cryobiology, Chapter 5, 127-147 (CRC Press, Boca Raton, FL, 1991)), and has been the predominant technique used in clinical practice. In early 1983, Stiff and colleagues developed a modified method for the preservation of stem cells (Stiff et al., Cryobiology, 20, 17-24 (1983)). In this method, the concentration of DMSO was reduced to 5% and an additional cryoprotective agent, hydroxyethyl starch ("HES"), was added to the solution. The cells could be frozen using a controlled rate freezer or in a mechanical freezer at -80°C.

In present clinical practice, the base of most cryopreservation solutions is a tissue culture medium, potentially containing many different components. Typical tissue culture media include RPMI 1640, IMDM, AIM-5, X-VIVO 10, or α MEM.

The cell suspension is then frozen, i.e., at liquid nitrogen temperatures in a suitable container and stored until needed. The frozen suspension is then thawed, by immersing the container in a warm water bath and culturing can be resumed. The resulting EC growth is similar to that of cells that were not subjected to cryopreservation.

The cultured EC may also be used in gene therapy wherein a gene producing a protein polypeptide, enzyme or other product is inserted into the DNA of the EC. The transgenic EC are then administered to a mammal, e.g., by infusion into a patient's bloodstream. See, for example, B. P. Luskey et al., Annals. N. Y. Acad. Sci., 612, 398 (1990), B. A. Naughton et al. (U.S. Pat. No. 4,721,096), and Anderson et al. (U.S. Pat. No. 5,399,346).

The gene carried by the EC can be any gene which allows the blood cells to exert a therapeutic effect that they would not ordinarily have, such as a gene encoding a clotting factor, such as Factor VIII, useful in the treatment of hemophilia. The gene can encode one or more products having therapeutic effects. Examples of suitable genes include those that encode cytokines such as TNF, interleukins (interleukins 1-12), interferons (α, β, γ-interferons), T-cell receptor proteins and Fc receptors for antigen-binding domains of antibodies, such as immunoglobulins.

Additional examples of suitable genes include genes that modify EC to "target" a site in the body to which the blood cells would not ordinarily "target," thereby making possible the use of the blood cell's therapeutic properties at that site. In this fashion, blood cells such as TIL can be modified, for example, by introducing a Fab portion of a monoclonal antibody into the cells, thereby enabling the cells to recognize a chosen antigen. Likewise, blood cells having therapeutic properties can be used to target, for example, a tumor, that the blood cells would not normally target. Other genes useful in cancer therapy can be used to encode chemotactic

factors which cause an inflammatory response at a specific site, thereby having a therapeutic effect. Other examples of suitable genes include genes encoding soluble CD4 which is used in the treatment of HIV infection and genes encoding α-antitrypsin, which is useful in the treatment of emphysema caused by α-antitrypsin deficiency, or genes encoding factors which promote bone growth such as bone morphogenic proteins (BMPs) or other factors in the BMP pathway.

The gene therapy of the present invention is useful in the treatment of a variety of diseases including but not limited to adenosine deaminase deficiency, sickle cell anemia, thalassemia, hemophilia, diabetes, α-antitrypsin deficiency, brain disorders such as Alzheimer's disease, and other illnesses such as growth disorders and heart diseases, for example, those caused by alterations in the way cholesterol is metabolized and defects for the immune system, as well as to repair bone fractures or treat or prevent osteoporosis.

In still another embodiment, there is provided a method of detecting the presence of human CEC present in a patient, comprising: (i) inserting into an expanded population of human CEC removed from the patient a DNA segment encoding the detectable marker under conditions such that the marker is expressed in the CEC; (ii) introducing cells resulting from step (i) into the patient; (iii) removing from the patient an aliquot of tissue (which can be, for example, normal tissue, cancerous tissue, vascular tissue, blood, lymph nodes, etc.) including cells resulting from step (ii) and their progeny; and (iv) detecting or determining the quantity of the cells resulting from step (ii) and their progeny, in said aliquot.

To effect gene therapy with a substantially pure population of human EC, the following general method may be used to insert a gene into these EC. For a review of transformation methodologies, see Friedman, Science, 244, 1275 (1989) and Lancet, Jun. 4, 1988, p. 1271. In order to introduce a normal structural gene, to correct a genetic error, a gene can first be isolated from the cells of a donor. The cells may be isolated from tissue(s), blood or other body fluids. To find a gene coding for the defective protein, DNA from the donor cell is isolated and cleaved by enzymatic digestion into segments of varying length by means known to those

skilled in the art. The segments of DNA then may be inserted individually into vectors containing the appropriate regulatory sequences for expression of a gene product. The vectors then can be screened by conventional means such as Northern blotting, if the sequence for the normal gene is known, or the expression product can be screened by Western blotting.

Alternatively, if the DNA sequence of the desired gene or the sequence of the normal protein is known, the gene can be made by synthetic methods such as by using a DNA synthesizer (Applied Biosystems). In any case, the method of isolation or construction of the gene sequence can yield a "normal" gene that codes for the desired gene product. Once isolated, a functional structural gene, such as a DNA sequence coding for a factor VIII protein, human factor VIII, porcine factor VIII and the like can be modified for expression *in vivo* by linkage to suitable control regions, such as promoters. Hybrid or modified genes can also be constructed, such as DNA sequences encoding factor VIII molecules, modified to reduce their antigenicity and immunogenicity. DNA methods can be used to substitute elements of animal factor VIII for the corresponding elements of human factor VIII, resulting in hybrid human/animal factor VIII molecules. The preparation of transformation vectors comprising DNA sequences encoding chimeric hybrid Factor VIII molecules is disclosed in Lollar et al. (U.S. Pat. No. 5,744,446). DNA methods may also be used to substitute amino acid residues at selected sites, such as epitopes, to yield factor VIII constructs having improved therapeutic attributes such as reduced antigenicity or immunogenicity or greater potency in comparison to unmodified human factor VIII.

In a preferred embodiment, a hybrid human/porcine cDNA encoding factor VIII, in which the porcine sequence encodes a domain or part domain, such as the A2 domain or part domain, is inserted in a mammalian expression vector, such as ReNeo, to form a hybrid factor VIII construct. Preliminary characterization of the hybrid factor VIII is accomplished by insertion of the hybrid cDNA into the ReNeo mammalian expression vector and transient expression of the hybrid protein in COS-7 cells. A determination of whether active hybrid protein is expressed can

then be made. The expression vector construct is used further to stably transfect cells in culture, such as baby hamster kidney cells, using methods that are routine in the art, such as liposome-mediated transfection (Lipofectin™, Life Technologies, Inc.). Expression of recombinant hybrid factor VIII protein can be confirmed, for example, by sequencing, Northern and Western blotting, or polymerase chain reaction (PCR). Hybrid factor VIII protein in the culture media in which the transfected cells stably expressing the protein are maintained can be precipitated, pelleted, washed, and resuspended in an appropriate buffer, and the recombinant hybrid factor VIII protein purified by standard techniques, including immunoaffinity chromatography using, for example, monoclonal anti-A2-Sepharose™.

In a further embodiment, the hybrid factor VIII comprising subunit, domain, or amino acid sequence substitutions is expressed as a fusion protein from a recombinant molecule in which a sequence encoding a protein or peptide that enhances, for example, stability, secretion, detection, isolation, or the like is inserted adjacent to the factor VIII encoding sequence. Established protocols for use of homologous or heterologous species expression control sequences including, for example, promoters, operators, and regulators, in the preparation of fusion proteins are known and routinely used in the art. See Current Protocols in Molecular Biology (Ausubel, F. M. et al., eds.), Wiley Interscience, New York.

The purified hybrid factor VIII or fragment thereof can be assayed for coagulation activity by standard assays including, for example, the plasma-free factor VIII assay, the one-stage clotting assay, and the enzyme-linked immunosorbent assay using purified recombinant human factor VIII as a standard. Antigenicity and immunogenicity of factor VIII constructs may be tested *in vitro* using the standard Bethesda inhibitor assay, or *in vivo* using a knockout hemophilic mouse model.

Other vectors, including both plasmid and eukaryotic viral vectors, may be used to express a recombinant gene construct in eukaryotic cells depending on the preference and judgment of the skilled practitioner. Other vectors and expression

systems, including bacterial, yeast, and insect cell systems, can be used but are not preferred due to differences in, or lack of, glycosylation.

The same methods employed for preparing hybrid human/porcine factor VIII having subunit, domain, or amino acid sequence substitution can be used to prepare other recombinant hybrid factor VIII protein and fragments thereof and the nucleic acid sequences encoding these hybrids, such as human/non-human, non-porcine mammalian or animal/animal. As used herein the term "Factor VIII protein" includes any of these materials that are biologically active as determined by the assays listed above, i.e., that possess coagulation activity when assayed *in vitro*. The murine and part of the porcine factor VIII cDNA have been cloned. Factor VIII sequences of other species for use in preparing a hybrid human/animal or animal/animal factor VIII molecule can be obtained using the known human and porcine DNA sequences as a starting point. Other techniques that can be employed include PCR amplification methods with animal tissue DNA, and use of a cDNA library from the animal to clone the factor VIII sequence.

Once the DNA containing the gene is prepared, the DNA can be inserted into the population of EC isolated and expanded as above. The DNA can be inserted by 1) physical methods such as coprecipitation with calcium phosphate, electroporation, lipofection or microinjection (e.g., U.S. Pat. No. 4,873,191), and/or by 2) the use of viral vectors such as adenoviruses, if the DNA is less than approximately 7-8 kB, or retroviruses for longer segments of DNA. In the latter case, the DNA of the retrovirus is cut with a restriction enzyme and the human DNA containing the desired sequence is inserted and ligated. The retrovirus containing the insertion then is transfected into the EC. The EC then can be assayed for production of the desired protein. See, e.g., U.S. Pat. Nos. 4,902,783 and 5,681,746.

In general, molecular DNA cloning methods are well known in the art and are not limiting in the practice of this invention. For a further description of similar methods, see Friedmann, Science, 244, 1275 (1989) and Molecular Cloning: A Laboratory Manual (2nd ed.), Cold Spring Harbor Laboratory Press, Sambrook, Fritsch and Maniatis eds. (1989).

Transgenic, i.e., transduced, endothelial cells stably incorporating and expressing heterologous DNA or RNA and therapeutic uses therefore, are described in Mulligan et al. (U.S. Pat. No. 5,674,722). In particular, retroviral vectors have been used to stably transduce endothelial cells with genetic material which includes genetic material encoding a polypeptide or protein of interest not normally expressed at biologically or therapeutically significant levels in endothelial cells. The genetic material introduced in this manner can also include genetic material encoding a dominant selectable marker, such as antibiotic or herbicide resistance. Genetic material including DNA encoding a polypeptide of interest alone, such as a Factor VIII protein, or DNA encoding a polypeptide of interest and a dominant selectable marker can be introduced into cultured endothelial cells. Expression of these genes by the endothelial cells into which they have been incorporated (i.e., endothelial cells transduced by the use of retroviral vectors) has also been demonstrated.

Endothelial cells transduced *in vitro* with the genetic material can then be transplanted using one of a variety of known methods. Such methods include, but are not limited to, the transplantation of synthetic vessels or prosthetic valves lined with transduced endothelial cells or the transplantation of a device or matrix designed to house transduced endothelial cells.

To administer the EC containing the desired gene, the cells may simply be introduced into the bloodstream of the patient by conventional means, such as of intravenous infusion over a period of time.

Endothelial cells which have been transduced *in vitro* are particularly useful for improving prosthetic implants (e.g., vessels made of synthetic materials such as Dacron®, Gortex® and other plastics or metal-plastic laminates), including shunts, stents and grafts, which are used in vascular reconstructive surgery. For example, prosthetic arterial grafts are often used to replace diseased arteries which perfuse vital organs or limbs. However, the currently available grafts are usually made of synthetic material and are subject to many complications, the worst of which is a high rate of thrombosis or occlusion. Animal studies suggest that lining the graft

with autologous endothelial cells prior to implantation may decrease, but not prevent, graft reocclusion with its attendant morbid consequences.

However, endothelial cells can be modified according to the method of the present invention in a way that improves their performance in the context of an implanted graft or provides a means for local drug delivery. Examples include local delivery to the interior of the lumen of antirestenotic, antiproliferative, or thrombolytic agents to prevent intraluminal clot formation, secretion of an inhibitor of smooth muscle proliferation to prevent luminal stenosis due to smooth muscle hypertrophy, and expression and/or secretion of an endothelial cell mitogen or autocrine factor to stimulate endothelial cell proliferation and improve the extent or duration of the endothelial cell lining of the graft lumen. The latter agents are termed "biocompatibilization" agents (polypeptides or proteins).

For a similar application, endothelial cells of the present invention can also be used to cover the surface of prosthetic heart valves to decrease the risk of the formation of emboli by making the valve surface less thrombogenic.

Endothelial cells transduced by the method of the subject invention or a vascular implant lined with transduced endothelial cells can also be used to provide constitutive synthesis and delivery of polypeptides or proteins, which are useful in prevention or treatment of disease, such as Factor VIII proteins, to treat hemophilia. In this way, the polypeptide is secreted directly into the bloodstream of the individual, from circulating cells, over an extended period of time. Currently available methods, in contrast, involve parenteral administration of the desired polypeptide.

In addition, there is no need for extensive purification of the polypeptide before it is administered to an individual, as is generally necessary with an isolated polypeptide (e.g., insulin). Endothelial cells modified according to the present invention produce the polypeptide hormone as it would normally be produced.

Another advantage to the use of genetically engineered endothelial cells is that one can target the delivery of therapeutic levels of a secreted product to a specific organ or limb. For example, a vascular implant lined with endothelial cells

transduced *in vitro* can be grafted into a specific organ or limb; or the endothelial cells of a particular limb, organ or vessel can be transduced *in vivo*. The secreted product of the transduced endothelial cells will be delivered in high concentrations to the perfused tissue, thereby achieving a desired effect to a targeted anatomical location. This product will then be diluted to nontherapeutic levels in the venous circulation during its return to the heart.

Another important advantage of the delivery system of this invention is that because it is a continuous delivery system, the short half lives of hormone polypeptides is not a limitation. For example, the half life of human growth hormone (HGH) is approximately 19 minutes and parathyroid hormone, approximately 2½ to 5 minutes.

The invention will be further described by reference to the following detailed examples.

Example 1

Culture of Endothelial Cells from Peripheral Blood

One hundred ml of fresh venous blood anti-coagulated with either heparin or citrate was diluted 1:2 with Hanks' Balanced Salt Solution (HBSS) containing 1 mM EDTA and 0.5% bovine serum albumin, carefully layered on an equal volume of Histopaque®-1077 (Sigma Chemical Co.) without disruption of the interface, and centrifuged at 400 × G for 30 minutes at room temperature. The layer containing the mononuclear cells was collected, and the cells were washed 3 times by centrifuging at 250 × G for 10 minutes, using the previously described MEC media (Gupta et al., Exp. Cell Res., 230, 244-251 (1997)) modified to contain 10% human male serum, to exclude the ECGS and comprising 1 µg/ml hydrocortisone. Washing with this culture medium is preferred over washing with buffer because the medium maintains higher levels of cell viability.

The buffy coat mononuclear cells were resuspended in EGM®-2 medium (Clonetics), and all buffy coat mononuclear cells from 100 ml of starting blood were placed into one well of a 6-well plate coated with collagen I (Becton-Dickinson).

This is defined as day #1, on which the plate is placed into an incubator at a controlled temperature (37°C) and humidified environment (5% CO_2). The EGM®-2 medium is changed daily. On day #2, most of the buffy coat mononuclear cells have remained unattached and they and cell debris were removed at the time of culture medium change. This left on the bottom of the culture well a small number of cells that have typical endothelial cell morphology and which stain positively with anti-endothelial monoclonal antibody P1H12. Typically, there were about 20-30 such cells identifiable on day #2, along with 100-200 other mononuclear cells.

After good cell growth was established (typically at about 20 days, when cells may or may not be confluent), they are passed into a fibronectin/gelatin-coated T25 flask, and then into fibronectin/gelatin-coated T75 flasks. To pass the cells, the wells are washed twice with calcium-free HBSS and then once with 0.5 X trypsin (i.e., 50% of the concentration for the Gibco BRL trypsin product recommended for cell lifting, because it is less harsh than the full-strength product) plus 1 mM EDTA. Cells detach after 2 minutes. Trypsin is neutralized by adding an equal volume of human serum or MEC media containing 20% human serum. Cells were collected from the solution by centrifugation at 250 × G for 5 minutes, and resuspended in EGM®-2 medium for continued culture.

Figure 1 is plot of the outgrowth of endothelial cells expanded using this method. The mean ± SD of five different culture experiments using five different blood donors was plotted up until culture passage 5, after which four different cultures were plotted. Error bars are not evident on the graph because of the high reproducibility of the method. As shown, the method of the invention can result in an outgrowth, and a 10^{18} fold expansion, of endothelial cells far greater than the 20-30 endothelial cells identifiable on culture day #2 (the day the unattached mononuclear cells and cell debris from the buffy coat are removed and any EC can be first identified).

It should be noted that this fold expansion is not a maximal expansion. It is just the degree of expansion allowed before cells are used for further experiments.

The maximal cap (if any) in expandability of these cells, therefore, has not yet been established or reached in these experiments.

The outgrowth cells have the characteristics of quiescent, microvascular EC. They have typical "cobblestone" endothelial morphology; they exhibit positive expression for von Willebrand Factor, P1H12 antigen, ICAM1, $\alpha_v\beta_3$, β_2-microglobulin, thrombomodulin, flk-1 (a VEGF receptor), and CD34; they are uniformly CD36-positive; they take up acetylated-LDL; they do not constitutively express tissue factor or VCAM, but do express both upon appropriate stimulation.

Example 2

Cyropreservation of Cultured Endothelial Cells

Vigorous EC outgrowth can be obtained even after cryopreservation. This is important because the utility of the present method is greater if cultured EC can be cryopreserved for later use.

To show this, EC were cryopreserved after their outgrowth had reached the capacity of two T75 flasks. To do this, the cells were detached as above and washed twice with HBSS. Cells were resuspended in 100% fetal calf serum at a concentration of one-million cells per 950 µl serum, and then 50 µl dimethylsulfoxide was added. The suspension was mixed quickly and placed on ice for 5 minutes. Afterward, the cells were stored at -70°C overnight, and they were then transferred to liquid nitrogen.

After six weeks of storage, the cells were thawed and one million cells were plated onto T75 flask with excess (20 ml) of EGM®-2 medium. Medium was changed again four hours after initial plating. The cells were then grown as in Example 1.

The resulting growth is similar to that of EC that were not subjected to cryopreservation, as shown in Figure 2. Again, data are shown as mean ± SD for three experiments. Cell count at origin indicates the number of cells thawed and started with. These data suggest that the endothelial outgrowth cells expanded from blood resume their vigorous growth rate after cryopreservation.

Example 3

Study of Origin of CEC in Blood

The origin of the CEC identifiable in fresh blood is not known, nor is the relationship between CEC and the endothelial outgrowth from cultured blood. To answer this, 4 adults were studied who had undergone allogeneic marrow transplantation (for malignancy) 5-20 months earlier using opposite-gender donors (three female donors to three male recipients and one male donor to one female recipient). All 4 subjects are disease-free and have peripheral blood and/or marrow aspirates that are 100% donor by RFLP; two also had marrow cytogenetics showing 100% donor.

The CEC in fresh blood, plus the peripheral blood endothelial outgrowth from buffy coat mononuclear cells cultured with endothelial growth factors was studied using the culture conditions of Ex. 1. MAb P1H12 was used to identify cells as being endothelial, and fluorescence *in situ* hybridization was used to identify cells as having XX or XY genotype.

CEC in fresh blood were almost exclusively of recipient genotype (95, 100, 100, 88%), arguing for their origin from the vessel wall. However, the blood endothelial outgrowth at 26-28 days (after 100-fold expansion) in culture was mostly donor genotype (82, 86, 77, 85%), revealing predominant origination from a marrow-derived cell. Remarkably, endothelial outgrowth at only 9 days (after 5-fold expansion) in contemporaneous culture was still largely recipient genotype (78, 88%). These data indicate that the CEC detected in fresh blood are derived from vessel wall and have some, but limited, expansion potential. Conversely, blood contains transplantable marrow-derived cells that take longer to expand but have greater proliferative potential. Thus, the CEC comprise a more mature and differentiated cell population, while there also are circulating marrow-derived endothelial-generating cells that comprise a more primitive progenitor population (putative angioblasts).

Example 4

Transfection of CEC with Factor VIII Expression Vectors

Endothelial cells were expanded from blood, as described in Example 1. For these transfection experiments, they were used after 4-5 passages. They were plated in a 6 well plate at density of 2×10^5/well, and allowed to attach overnight. Then, cells in different wells were transfected by lipofection with one of four vectors as summarized in Table 1, below.

Table 1. Plasmid Vectors for CEC Transfection

A. Two vectors from Octagen with B-domainless FVIII replaced with green fluorescent protein (GFP):
   #1 HSQ/eGFP/ReNeo
   #2 HSQ/eGFP/CP

B. Control vector from Octagen with GFP but no factor VIII:
   #3 pEGFP-N1

C. Vector constructed at University of Minnesota, Department of Medicine with the FVIII/GFP from Octagen inserted between XhoI and NotI sites in commercial vector pcDNA3.1(-):
   #4 pcHSQ/eGFP

All the transfection vectors contain the neomycin resistance gene (ReNeo) as a selection marker.

Vectors 1, 2 and 4 contain an HSQ/eGFP insert which codes for a fVIII-dGFP fusion protein. This insert comprises DNA that codes in order of sequence, 1) the human fVIII human activation peptide, 2) the human fVIII A1 domain, 3) the human fVIII A2 domain, 4) the first five amino acids of the SQ B domain linker peptide, 5) the enhanced green fluorescent protein (eGFP), 6) the last nine amino acids of the SQ linker peptide, 7) the human fVIII light chain activation peptide, 8) the human fVIII A3 domain, 9) the human fVIII C1 domain, and 10) the human fVIII C2 domain.

The DNA sequence of HSQ/eGFP is depicted in Fig. 3 (SEQ ID NO:1). The DNA sequence contains 5094 bases, encoding the peptides listed on Table 2.

Table 2. HSQ/eGFP Peptides SEQ ID NO:1

1-57 signal peptide
58-1173 A1 domain
1174-2274 A2 domain

2275-2292 SQ sequence (first part)
2293-3012 eGFP sequence
3013-3039 SQ sequence (second part)
3040-3162 light chain activation peptide
3163-4152 A3 domain
4153-4611 C1 domain
4612-5091 C2 domain
5092-5094 stop codon

ReNeo is a mammalian expression vector (non-proprietary). The second plasmid, HSQ/eGFP/CP, contained the same fVIII construct but the vector is the same one used to express eGFP (see below). The eGFP protein was removed from p-EGFP-N1 and replaced with the HSQ/eGFP construct. The third plasmid is p-EGFP-N1 from Clontech, which expresses only eGFP.

The transfection protocol was based on the instructions supplied by Life Technologies Co. with the lipofectamine. Briefly, 2 micrograms of plasmid DNA was mixed gently with 15 microliters of lipofectamine in 200 microliters of E-STIM® basal medium (Becton Dickinson) without antibiotics. The mixture was allowed to stand at room temperature for 30 minutes. Expanded endothelial cells in 6-well plates were washed twice with E-STIM® basal medium before adding the DNA-liposome complex. Dropwise, 1.8 ml of E-STIM® was added to the DNA-liposome complex, then the mixture was overlaid on the endothelial cells. After 5 hours incubation, the DNA-liposome mixture was replaced with the endothelial culture medium of Example 1. Then, 2 days after transfection, the cells from one culture well were subcultured into one 10 cm dish in endothelial culture medium containing 50-100 μg/ml of Geneticin (Life Technologies Co.). This selection medium was changed every other day.

Transfection of endothelial cells derived from the blood of two donors has been accomplished to date. The number of endothelial colonies (derived from the transfection of greater than 2 x 10^5 cells) was checked on day 20 in selection medium for two donors, as shown on Table 3.

Table 3. Number of Colonies After 20 Days in Selection Medium

Vector #	Donor 1	Donor 2
1	3	9
2	2	8
3	4	7
4	1	8

On day 20, fluorescence microscopy showed all selected colonies to be positive for green fluorescent protein (GFP). On day 20, the colonies were pooled and transferred to the regular endothelial growth condition.

In the past, many basic and applied studies had to be performed on endothelial cells from other animal species because existing culture techniques permitted only restricted proliferation of human endothelial cells. The present method accomplishes at least a 10^{18}-fold expansion of circulating human EC. This method will permit peripheral blood from living donors to be used for the generation of large numbers of cultured endothelial cells. Thus, problems of human pathology involving the endothelium now can be approached directly by employing a human endothelial cell model. In addition, expanded mammalian EC should prove valuable for various clinical applications, such as *in vitro* testing of vasoactive agents, the coating of artificial graft materials and gene therapy designed to treat vascular pathologies and genetic disorders, for local drug delivery and enhanced biocompatibility.

Example 5

Use of Endothelial Cell Culture Methodology in Disease Diagnostics

Currently, there is no direct or non-invasive method to examine a patient's endothelium to assess it for either acquired or genetic defects that contribute to disease. A 25 year old male patient had been evaluated for the presence of a hypercoagulability disorder. The patient had a history of multiple thromboses, and a family history suggesting a familial pre-disposition to thrombosis. No positive diagnosis was obtained after extensive evaluation for known genetic and

predisposing causes. Therefore, the patient was evaluated for a defect in thrombomodulin. Thrombomodulin is an enzyme that is expressed on the endothelial cell surface and is an anti-thrombotic defense. A patient having a mutation in thrombomodulin that leads to poor function would have an increased risk for thrombosis.

Endothelial cells were expanded from the blood of the patient and three normal control donors using the present method. Thrombomodulin activity was assessed in a standard biochemical assay. Briefly, a known number of endothelial cells is incubated with bovine protein C (100 µL per well of a 1 µM solution) and human recombinant thrombin (10 µL of 10 nM). It is incubated at 37°C for 2.5 hours. The reaction is terminated by adding EDTA amd I-2581 (Chromogenix AB, Molndal, Sweden), and the chromatographic substrate S-2366 (DiaPharma Group, Inc., Franklin, OH) is added. Supernatant absorbance is read at 405 nm.

The results are given in OD units per minute per 50,000 endothelial cells. The value for 18 samples from the three normal donors was 53±7, while the value for six samples from the patient was 41±5, a significant difference (p=0.0034). Thus, the expanded endothelial cells from the patient had a significantly lower thrombomodulin activity than those of control patients. Therefore, a genetic mutation in thrombomodulin resulting in a thrombomodulin deficiency may be the underlying basis for this patient's disorder. Molecular biological analyses are employed to confirm the genetic basis for the thrombomodulin deficiency.

Example 6

Stable Transfection, Selection and Expansion

The plasmid pLE (Clontech), which contains sequence for enhanced green fluorescent protein (eGFP), was transfected into line PA317. Virus from one of the resulting cloned transfectants, pLE9, was employed to infect endothelial cells.

A culture of blood buffy coat mononuclear cells was set up according to the method of the invention. When cells had reached passage 4 and about a 10^6 fold expansion, they were seeded at 10^5 cells/dish, with the 10 cm dishes coated with 6

microgram/cm^2 of type 1 collagen and 250 microgram/dish of fibronectin in 1% gelatin. After two days of growth, the cells were exposed to conditioned medium from the packaging cell line in combination with 4 microgram/ml of polybrene to dishes that were 70% confluent. After three days exposure, cells were split 1:2 into a 10 cm dish (coated as above), and new culture medium added. Then they were subjected to selection in media containing G418 for 19 days.

As shown in Figure 5, cultured endothelial cells prepared according to the method of the invention can survive chemical selection and be expanded thereafter, and be stably transfected to express a foreign (non-selected) gene. Moreover, the outgrowth cells have preserved normal endothelial cell morphology.

Example 7

Transfection of CEC with Human Factor VIII (hFVIII) Expression Vectors

Materials and Methods

Transfection

Ten cm dishes and T75 flasks were coated with 6 µg/ml of type I collagen and 50 µg/ml of fibronectin. Passage 4 or 5 outgrowth endothelial cells were obtained as described in Example 1. They were seeded into the dish or flasks and allowed to grow to 40% confluence. Then the cells were transfected with hFVIII vectors (Table 4) using transfection reagent Fugene6 (Roche Molecular Biochemicals) following the manufacturer's protocol. Briefly, Fugene6 was added to each vector at a ratio of 2.5:1. Ten µg of DNA was used for transfection of cells in 10 cm dishes; and 15 µg of DNA was used for cells in T75 flasks.

The Fugene6-DNA mixture was added to the cells in media containing 10% human serum and incubated for 3 days. Cells reached 100% confluence after 3 days.

Table 4. Plasmid Vectors

pTracerHSQ: has HSQ (the B-domainless hFVIII without the eGFP insert) cloned into pTracer-CMV (Invitrogen)

pcF8G: has HSQ/eGFP (eGFP replaces the B domain of hFVIII) cloned into plasmid pCDNA 3.1 (Invitrogen)

pcHSQ: has HSQ cloned into plasmid pCDNA 3.1 (Invitrogen)

pTracerCMV: obtained from Invitrogen

HSQ/GFP/ReNeo: has HSQ/eGFP cloned into pRENeo (Biogen)

Transient expression

At the end of 3 days, the conditioned media from T75 flasks were collected and centrifuged. The presence of hFVIII in the supernatant was detected using an ELISA kit (American Diagnostics). Cells from T75 flask were harvested, lysed and processed (RT-PCR, Titan One Tube RT-PCR System, Roche Molecular Biochemicals) for detection of hFVIII mRNA. A reverse transcriptase reaction was conducted at 50°C for 45 minutes, then the samples were subject to 94°C, for 2 minutes; 10 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute; 20 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, plus cycle elongation of 5 seconds for each cycle elongation at 72°C for 7 minutes. The primer pair employed was hFVIIIC-S (5' GCC CTT TTC TTG GAT CAA GGT GG3'; SEQ ID NO: 3) and hFVIIIC-AS (5' CTC CCT GAG TAG TTA CTC CTG TG3'; SEQ ID NO: 4).

Results

Three days after infection, hFVIII was detected in the supernatant of transfected cells (Figure 6) but not in control cells. Figure 7 shows the results of a RT-PCR analysis of RNA from those cells. The expression of hFVIII mRNA is increased in transfected cells relative to control cells.

At the end of 3 days, cells from 10 cm dishes were split into 2 10 cm dishes coated with 6 µg/ml of type I collagen and 50 µg/ml of fibronectin. The following day, cells were exposed to selection medium EGM-2 (Clonectis) containing 50 to 100 µg/ml of G418 or 50 µg/ml of Zeocin or both (Zeocin was used for selection of

pTracerHSQ; G418 for pcFVIIIG and HSQ/GFP/ReNeo; and both Zeocin and G418 were used for cells subjected to cotransfection). Stable expression of hFVIII is detected in these cells after chemical selection.

All patents, patent applications and publications cited hereinabove, are incorporated by reference herein. While a preferred embodiment of the present invention has been described, it should be understood that various changes, adaptations and modifications may be made therein without departing from the spirit of the invention and the scope of the appended claims.